WOMEN IN CANCER THEMATIC REVIEW

Systemic therapies in neuroendocrine tumors and novel approaches toward personalized medicine

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Abstract

Neuroendocrine tumors (NETs) are a group of heterogenous neoplasms. Evidence-based treatment options for antiproliferative therapy include somatostatin analogues, the mTOR inhibitor everolimus, the multiple tyrosine kinase inhibitor sunitinib and peptide receptor radionuclide therapy with 177-Lu-octreotate. In the absence of definite predictive markers, therapeutic decision making follows clinical and pathological criteria. As objective response rates with targeted drugs are rather low, and response duration is limited in most patients, numerous combination therapies targeting multiple pathways have been explored in the field. Upfront combination of drugs, however, is associated with increasing toxicity and has shown little benefit. Major advancements in the molecular understanding of NET based on genomic, epigenomic and transcriptomic analysis have been achieved with prognostic and therapeutic impact. New insight into molecular alterations has paved the way to biomarker-driven clinical trials and may facilitate treatment stratification toward personalized medicine in the near future. However, an improved understanding of the complexity of pathway interactions is required for successful treatment. A systems biology approach is one of the tools that may help to achieve this endeavor.

Introduction

Neuroendocrine tumors (NET) represent a diverse group of rare neoplasms, sharing features of endocrine and neuronal cells. The incidence is 2.5–5/100,000 population, and is still increasing (Yao *et al.* 2008*a*). Most frequently, they occur in the gastrointestinal (GI) tract and pancreas, followed by the lungs. Due to their indolent course, they are often diagnosed at an advanced tumor stage, particularly if localized in the small intestine or pancreas (Lawrence *et al.* 2011). From a clinical perspective, NET can be divided into functional and non-functional tumors.

Functional tumors account for one-third of all NET and are characterized by specific symptoms associated with an elevation of specific biomarkers (e.g. carcinoid syndrome in serotonin-secreting tumors, hypoglycemia in insulin-producing pancreatic NET). In the current WHO classification, NET are divided based on their histology including grading, i.e. proliferative activity (Ki-67 in % and/or mitotic count/10 high power fields) in NET G1 (≤2% Ki-67 or ≤2 mitoses), NET G2 (≥3–20% Ki-67 or <20 mitoses) and small-cell or large-cell neuroendocrine

carcinoma (NEC G3; >20% Ki-67 or >20 mitoses) (Bosman *et al.* 2010). One of the common features of NET G1/G2 is the expression of somatostatin receptors (sstr) that can be exploited for functional imaging (octreoscan or 68-Ga-DOTA-PET/CT) and somatostatin receptor-targeted therapy. In contrast, sstr expression in NEC G3 is rather low (van Essen *et al.* 2014). Several parameters have an impact on the prognosis and therapeutic management, most importantly Ki-67 and primary site, but also tumor burden, age at diagnosis and WHO performance status (Durante *et al.* 2009, Frilling *et al.* 2012, Rindi *et al.* 2012, Rinke *et al.* 2016). On the one hand, evidence-based medicine has changed the treatment landscape in the last 5–7 years. On the other hand, clinical and pathological criteria still guide therapeutic decisions in the absence of definite molecular predictors of response. Although several approved and non-approved drugs are available for systemic treatment, patients may develop resistance to therapy or show intrinsic resistance. The identification of predictors of tumor response is urgently required to improve the outcome and avoid unnecessary toxicity of ineffective therapies. This review summarizes established treatment options in advanced GI and pancreatic NET with a focus on antiproliferative therapies, recent endeavors to improve the outcome and novel approaches toward a more personalized medicine.

Current treatment options: what is evidence based?

Therapeutic options include surgery, loco-regional and ablative therapies, somatostatin analogues, interferon alpha, novel targeted drugs, peptide receptor radionuclide therapy (PRRT) and systemic chemotherapy. All options are established in the management of NET, but their evidence level differs for intestinal (iNET) and pancreatic NET (pNET) (Fig. 1). Formerly the term 'carcinoid' was used not only for intestinal NET but also for NET of other origin, such as gastric, lung, thymic and other less frequent types of NET. Although the term 'carcinoid' was replaced by NET in the WHO classification of GI NET (Bosman *et al.* 2010), it is still preserved for lung carcinoids.

Surgery is the mainstay of treatment for functionally active NET and should even be considered in non-curative resectable metastatic disease to alleviate symptoms, particularly in metastatic pNET such as insulinoma or NET associated with the carcinoid syndrome. If the general condition of the patient is appropriate, non-functioning NET G1 and G2 should be considered for curative resection according to expert recommendations (ENETS consensus guidelines and NCCN guidelines) (Kulke *et al.* 2015*d*, Pavel *et al.* 2016). However, all studies are retrospective,
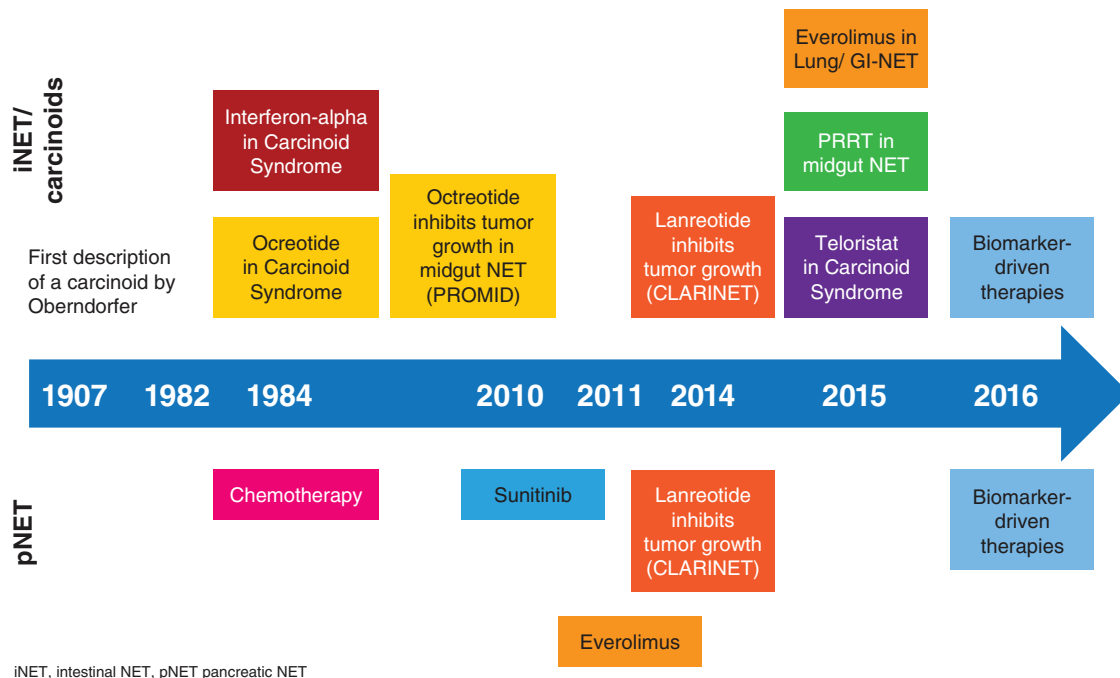

Figure 1
Evolution of therapies in neuroendocrine tumors.

and up to 90% of patients have recurrent disease within 5 years if liver metastases existed before surgery (Frilling et al. 2012). A systematic review found no robust evidence that a liver resection was superior to any other liver-directed therapies in improving overall survival or progression-free survival (PFS) (Lesurtel et al. 2015). Histology, including Ki-67, tumor extent and presence of extra-abdominal disease, limits the use of surgery (Tamburrino et al. 2016).

Somastostatin analogues (SSA) are the mainstay of therapy for symptoms related to the carcinoid syndrome and functionally active pNET, such as VIPOMA and glucagonoma since the mid-1980s. Two commercially available SSA, octreotide and lanreotide, preferentially bind to sstr-2 and to a lesser extent to sstr-5 that are expressed in 80–90% of well-differentiated G1/G2 NET; SSA are available as long-acting release formulations; octreotide is also available as short-acting sc. formulation (Modlin et al. 2010). In two placebo-controlled trials (PROMID and CLARINET), the antiproliferative activity has been demonstrated by the prolongation of time to progression (TTP) and PFS, respectively for octreotide in low-grade (G1) midgut NET (TTP 14.3 months vs 6 months on placebo) and for lanreotide in entero-pancreatic NET (PFS >27 months vs 18 months on placebo) of low to intermediate grade (G1 and G2 ≤10% Ki-67) (Rinke et al. 2009, Caplin et al. 2014). Although objective remissions are rare with SSA (<2%), disease stabilization is observed in two-thirds of the patients. Lanreotide was active irrespective of Ki-67, tumor burden and primary site. The open-label extension study of the CLARINET study confirmed the antiproliferative activity in progressive disease patients on placebo who crossed over to open-label lanreotide (Caplin et al. 2016). Most patients had stable disease before therapy in the CLARINET study, whereas the disease status was unknown in the PROMID study. As in the CLARINET study patients on the placebo arm remained stable for a long time (median PFS in small intestinal (SI) NET 21 months, in pNET 12 months), a watch-and-wait strategy may be justified in a subgroup of patients that still needs to be defined at a molecular level. Although the somatostatin receptor as a target is broadly expressed in NET, there are no predictors of response. Patients with low Ki-67 (<5% or <10%) have probably a more durable benefit (Jann et al. 2013, Faggiano et al. 2016). Prospective data are lacking in NET G2 up to 20% Ki-67 but will be generated by an ongoing clinical trial (CLARINET forte, NCT02651987) in progressive GEP-NET. The expression of a truncated splice variant of sstr-5 was associated with poor biochemical response to octreotide in acromegaly (Marina et al. 2015). The finding that

tumor expression of the truncated sstr-5 was associated with more aggressive behavior in pancreatic NET warrants further investigation in NET (Sampedro-Núñez et al. 2016). The value of SSA for NET of other origin, such as gastric or rectal NET remains unclear, their use, however, seems justified based on the expression of somatostatin receptors, and low grade or slow growth in the absence of any approved drugs. Similarly, the role of SSA in lung NET (carcinoids) is not well defined; however, clinical trials (e.g. SPINET, NCT02683941) will assess their value in the future. SSAs have a well-known and favorable long-term safety profile (Rinke et al. 2009, Modlin et al. 2010, Caplin et al. 2014), and this supports consideration as a first-line therapy in different types of NET.

Interferon alpha (IFN-α) has been introduced in the management of carcinoid syndrome in the mid-1980s (Öberg et al. 2000). Interferon-α acts by binding to specific receptors on the cell surface, and subsequently activating cytoplasmic messengers, such as Janus kinase 1 (JAK-1) and tyrosine kinase 2 (TYK-2). IFN-α inhibits the secretion of bioactive compounds including serotonin and tumor growth by multiple effects including a direct inhibitory effect on the cell cycle, inhibition of growth factors production, antiangiogenic effects and modulation of the immune response (Platanias 2005). Due to more pronounced side effects compared with SSA, it is less frequently used, unless in refractory carcinoid syndrome, and for antiproliferative purpose in selected cases, e.g. in sstr-negative NET (Pavel et al. 2016). In a large randomized trial with more than 400 patients with carcinoids, IFN-α of 5 million units three times per week was equally effective as bevacizumab, each in combination with octreotide (Yao et al. 2015).

Peptide receptor radionuclide therapy (PRRT) either with 90-yttrium-labeled compounds or more recently with 177-Lu-DOTATATE has been used for 15 years in uncontrolled trials in different types of NET demonstrating mostly stabilization of disease and in subpopulations remissions in 15–35% of patients (Brabander et al. 2016). Recently, the results of the first randomized controlled trial in midgut NET (NETTER-1) with 4 cycles of Lu-DOTATATE and concomitant octreotide compared with high-dose octreotide (60 mg/month) after failure of standard dose of octreotide have been reported (Strosberg et al. 2015). The objective response rate was 19% with PRRT and 2% with high-dose octreotide. Median PFS with PRRT was not reached (>27 months) and was 8.4 months with octreotide. Expression of sstr is a prerequisite for the use of PRRT. It remains unclear which patients have the highest benefit with respect to objective and durable

response; however, a strong expression of sstr-2 (Krenning Scale 3–4 as fulfilled in the NETTER-1 trial) seems to be of importance. Prospective trials to explore the role of PRRT in pNET and NET of other sites are ongoing (www.clinicaltrials.gov). Different drugs (temozolomide, capecitabine or everolimus) have been used in combination with PRRT; however, it remains unclear if combination therapies including the use of radiosensitizers will be superior to PRRT alone (Hubble et al. 2010, Claringbold et al. 2012, 2015), and further prospective clinical trials are warranted.

Among novel targeted drugs many different agents (tyrosine kinase inhibitors, anti-VEGF, IGF-R antibodies, EGFR antagonists and novel somatostatin analogues) have been explored in phase II trials (Pavel & Wiedenmann 2011); however, few drugs have advanced toward randomized controlled phase III trials, e.g. the mTOR inhibitor everolimus, and the multiple tyrosine kinase inhibitor sunitinib. Sunitinib and everolimus represent the only approved novel targeted drugs in NET. Everolimus has been most extensively studied in NET and has activity in a broad profile of patients with progressive disease. Components of the mTOR pathway are activated in NET, more frequently in pNET compared with SI NET (Kasajima et al. 2011). Based on prolongation of PFS, everolimus was first approved in pNET (median PFS 11 months with everolimus vs 4.6 months on placebo; RADIANT-3), and more recently in non-functional progressive intestinal and lung NET (median PFS 11 months with everolimus vs 3.9 months on placebo; RADIANT-4) (Yao et al. 2011b, 2016). In contrast, in a large trial of everolimus+octreotide in patients with carcinoid syndrome, the efficacy was less clear (Pavel et al. 2011). Around 60% of patients require dose reduction and 12–19% withdrew therapy in clinical trials due to side effects (Pavel et al. 2011, Yao et al. 2011b, 2016). Some side effects like infections may be crucial and require careful selection and surveillance of patients, thus strengthening the unmet need of predictive biomarkers.

Preclinical studies indicate that susceptibility to everolimus may vary in individual patients even if the tumor has the same site of origin (Svejda et al. 2011; Gagliano et al. 2013). As demonstrated in the RADIANT-4 study, everolimus was active in G1 and G2 NET (Yao et al. 2016). However, in most studies, no data of Ki-67 were available. Clinical trials exploring everolimus in G3 NEN are ongoing (EVINEC, NCT02113800, NCT02248012; www.clinicaltrials.gov). The prior use of chemotherapy had no impact on everolimus efficacy in pNET (Lombard-Bohas et al. 2015). The observation that 14% of patients

with pNET have mutations in the mTOR pathway (Jiao et al. 2011) while these mutations are extremely rare in intestinal NET, however, is in contrast to the finding that a clinical benefit in many patients with a disease control rate of 73 and 81% was observed with everolimus in pNET (RADIANT-3) and GI/lung NET (RADIANT-4) and a PFS benefit of 6 and 7 months, respectively. Downregulation of mTOR pathway components (e.g. TSC-2, PTEN), a frequent finding in pNET tumor samples, might be more indicative of tumor response although correlative data to everolimus efficacy are lacking (Missiaglia et al. 2010). Different variables have been tested for their predictive value (the occurrence of stomatitis, an early decrease of circulating chromogranin A and the FGFR4 genotype polymorphism), but none of them represents a definite predictor of response to everolimus (Yao et al. 2011a, Serra et al. 2012, Cros et al. 2016, Rugo et al. 2016).

Neuroendocrine tumors are known to be highly vascular and to express vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) in tumor and microenvironment. Sunitinib inhibits several tyrosine kinases, including the VEGF receptor and the PDGF receptor. Sunitinib is an approved therapy in progressive pancreatic NET based on prolongation of PFS by 6 months compared with placebo (median PFS 11.4 months with sunitinib vs 5.5 months on placebo; Raymond et al. 2011). Circulating cytokines (interleukin-8, soluble VEGFR-3 and stromal cell-derived factor-1α) and circulating tumor cells have been associated with outcome to sunitinib and pazopanib therapy (Ebos et al. 2007, Grande et al. 2015, Zurita et al. 2015), but none of these markers is validated or established in clinical practice. There is no definite evidence of efficacy of tyrosine kinase inhibitors in non-pancreatic NET yet, although phase II trials indicate some efficacy of novel tyrosine kinase inhibitors (pazopanib, axitinib) (Phan et al. 2015, Strosberg et al. 2016). Common side effects like hypertension or fatigue warrant careful selection of patients for therapy.

Systemic chemotherapy is used in pNET and NEN G3; less frequently in higher grade 2 intestinal NET (Pavel et al. 2016). Streptozotocin-based chemotherapy, either in combination with 5-FU or doxorubicin is an established therapy for pNET that has been approved in the US in the mid-1980s. It is especially used in G2 pNET if they are progressive or associated with higher tumor burden. Response rates of 25–42% from retrospective studies of different institutions support its activity in the era of novel targeted drugs (Dilz et al. 2015, Krug et al. 2015, Clewemar Antonodimitrakis et al. 2016).

Endocrine-Related Cancer

Response to chemotherapy increases with Ki-67 index, but Ki-67 alone is an unreliable means to select patients for chemotherapy (Childs *et al.* 2016). Temozolomide-based chemotherapy, either as monotherapy or in combination with capecitabine or bevacizumab may produce response rates of 30–70% based on a limited number of mostly retrospective studies and represents an alternative approach in pNET (Koumarianou *et al.* 2015). Pending data from a prospective trial of temozolomide vs temozolomide+capecitabine in progressive pNET (NCT01824875, www.clinicaltrials.gov) will provide more evidence for its use in the near future. There is low evidence for the use of chemotherapy in non-pancreatic GI NET (carcinoids) (Lamarca *et al.* 2016). In NEC G3 cisplatin or carboplatin+etoposide is standard treatment; however, responses are short lasting (in median 4–6 months) and overall survival (OS) is poor. There is no evidence for 2nd-line therapies in NEC G3 although based on small retrospective trials from single centers, different regimens may be considered (FOLFOX; FOLFIRI, temozolomide/capecitabine) (Garcia-Carbonero *et al.* 2016).

Two novel drugs have been explored for syndrome control in patients with carcinoid syndrome refractory to standard SSA dose. Pasireotide has a broader specificity and high affinity for sstr1-3 and sstr-5 compared with octreotide or lanreotide. In a comparative phase III trial, however, pasireotide (60 mg) was not superior to octreotide LAR (40 mg) every 28 days for syndrome control at 6 months (Wolin *et al.* 2015). Telotristat ethyl, previously named telotristat etiprate (the hippurate salt of telotristat ethyl), is an oral tryptophan hydroxylase inhibitor, a rate-limiting enzyme in the synthesis of serotonin, that has demonstrated in a phase III trial (TELESTAR) a significant improvement of bowel movements compared with placebo (Kulke *et al.* 2015*a*). If approved this innovative drug will fulfill an unmet need for a subpopulation of patients suffering from tumor-related serotonin hypersecretion (Pavel *et al.* 2015, Kulke *et al.* 2015*a*).

In the absence of any comparative trials of systemic therapies, the recently updated ENETS consensus guidelines take into consideration different clinical and pathological criteria next to the evidence level of drugs to guide therapy. Nevertheless, therapy selection is frequently a very individualized approach taking into account patient-related factors including comorbidities. The presence of an increasing number of agents explored in the field and the rarity of the disease make the identification of molecular predictors of response and construction of molecularly driven trials essential.

Limitations of clinical trials

There are different methodological or tumor-derived issues that limit the exploration of predictive biomarkers in clinical trials, among them the limitation to single biopsies and use of archival tissues, clinical trial design and morphological criteria of response assessment.

Tissue biomarkers

Findings on archival tissues from primary tumors or metastases may be misleading as they do not necessarily reflect molecular alterations in metastases at a later time point or after a series of systemic therapies. Further, a single biopsy may miss depiction of potential heterogeneity of tumors and thus influence the validation of biomarkers owing to sampling bias (Nash *et al.* 2012). Preclinical and clinical studies indicate that tumor heterogeneity exists in NET. Interestingly, in a PDGF-deficient knockout mouse, a functionally important malignant cell heterogeneity could be revealed in pNET (Cortez *et al.* 2016). Further, heterogeneity in NET is reflected by tumors with mismatch on functional imaging using FDG-PET and somatostatin receptor imaging. The combined assessment of genomics with functional imaging may represent a novel approach toward personalized cancer therapy (Basu & Basu 2016).

Patient selection and design

Patient homogeneity and rigorous design seem to be crucial for construction of an informative clinical trial (Halperin & Yao 2016). Some early comparative trials in the field of NET were underpowered (Faiss *et al.* 2003, Arnold *et al.* 2005). Some patients were probably included in clinical trials at very advanced disease stages; thus, the potential benefit of a drug might have been underestimated. As an example, the insulin-like growth factor-1 receptor inhibitor (MK-0646) failed to show objective responses in patients with metastatic well-differentiated NET. The poor outcome of patients with carcinoids (PFS 2.7 months, OS 10.5 months), however, indicates selection of a subpopulation with very poor prognosis (Reidy-Lagunes *et al.* 2012).

Assessment of tumor response in clinical trials

Most prospective clinical trials use RECIST criteria to assess tumor response. However, only very few trials used the same criteria to assess the tumor growth behavior accurately before enrolment in a clinical trial,

e.g. the CLARINET study and a phase II trial of temsirolimus and bevacizumab (Caplin *et al.* 2014, Hobday *et al.* 2015). If the accurate tumor growth rate is unknown before a new therapy, the tumor might potentially display the same growth rate but still would be considered as being stable according to RECIST as long as 20% tumor volume increase is not exceeded, and thus, the patient will be assessed as a responder. Assessment of the tumor growth rate is a novel approach to better determine response (Ferté *et al.* 2014). Central review of radiological scans that determine the slope of progression before enrolment should be implemented in clinical trials.

Current approaches to improve outcome

Overall, none of the available systemic treatments achieves cure, and objective response rates are low with targeted drugs (<5–10%). Prolongation of PFS is the primary endpoint in most trials. Limited response duration requires novel approaches to overcome resistance to drugs. Recent prospective randomized trials failed to demonstrate a clear survival benefit, what is in part due to the nature of the disease and high cross-over rate to open-label drug in most randomized trials (Yao *et al.* 2011*b*, Vinik *et al.* 2012, Caplin *et al.* 2014, Rinke *et al.* 2016). Different approaches to improve the outcome include combination of different drugs, the predetermined sequential use of drugs, and the use of less toxic drugs as a maintenance therapy after prior use of a more aggressive therapy. Combination of different drugs is the most frequently used approach.

Combination therapies

From a pathophysiological point of view, it is an attractive approach to combine two or three drugs targeting different pathways in NET. Multiple combination therapies have been explored in phase I/II trials, either as a de novo combination of two targeted drugs or of targeted drugs with somatostatin analogues, and also with chemotherapy, PRRT or even loco-regional therapies of the liver. Frequently, somatostatin analogues are kept as a basic therapy beyond progression while evidence of their add-on value to 2nd- or 3rd-line therapies for antiproliferative purposes is lacking (Dasari *et al.* 2015, Kulke *et al.* 2015*b*, Yao *et al.* 2015). Some drug combinations have shown unfavorable safety profile and have not been further explored, e.g. the multiple tyrosine kinase inhibitor sorafenib and bevacizumab, or everolimus, either with the anti-IGF1R antibody cixutumumab and octreotide or with

erlotinib (Bergsland *et al.* 2012, Castellano *et al.* 2013, Dasari *et al.* 2015).

Many of the phase II trials exploring combination therapies are single-arm trials, and do not provide definite evidence of a superiority of combination therapies. For example, there are no clear data of a favorable effect when combining bevacizumab with streptozotocin in pNET or with oxaliplatin in different types of NET such as carcinoids, pancreatic NET or poorly differentiated NEC (Ducreux *et al.* 2014, Kunz *et al.* 2016).

Previous randomized controlled trials that investigated SSA, either octreotide or lanreotide with IFN-α failed to demonstrate the superiority of the combination therapy compared with monotherapy, but were underpowered and enrolling heterogeneous patient populations. The use of the combination of SSA with IFN-α was associated not only with higher response rates but also with increasing toxicity (Faiss *et al.* 2003, Arnold *et al.* 2005) (Table 1). Discontinuation of treatment as a result of side effects occurred in 20% of patients receiving the combination of octreotide and IFN-alpha and in 4% of patients receiving octreotide monotherapy (Arnold *et al.* 2005).

The few drug combinations that were investigated in comparative well-designed randomized controlled trials in progressive pNET failed to demonstrate that the combination of drugs was clearly superior to monotherapy. In one of these trials, everolimus was explored in combination with a novel somatostatin analogue, pasireotide that binds to four of five somatostatin receptors and downregulates IGF-1, an upstream activator of the mTOR pathway (Kulke *et al.* 2015*c*). Another trial investigated the combination of everolimus and an angiogenesis inhibitor, bevacizumab, which binds circulating VEGF (Kulke *et al.* 2015*b*). Further the dual PI3 kinase mTOR inhibitor BEZ235 was studied to inhibit potential upstream activation of the mTOR pathway along with mTOR inhibition (Libutti *et al.* 2015) (Table 1). Although the improvement in PFS seen with the combination of everolimus and bevacizumab vs everolimus alone met the predefined statistical criteria ($P<0.15$), the PFS benefit was modest (2.7 months) and was achieved at the expense of more side effects; the other two studies failed to show any benefit in PFS. However, it should be emphasized that subpopulations of patients achieved objective response of 20 and 31% in the trials of everolimus with pasireotide or with bevacizumab, respectively. Under the condition that higher objective response rates translate into more durable response, these combinations might still be considered in individual

Table 1 Combination therapies in NET in randomized controlled trials.

Study	Drugs	Tumor type	Patients	Objective response (%)	Median PFS[d] (months)	Overall survival (months)
Faiss et al. (2003)	LAN	GEP-NET	80	4	NS (TTP)	NA
	IFN			3.7		
	LAN+IFN			7.1		
Arnold et al. (2005)	OCT	GEP -NET	109	2	NS	35
	OCT+IFN			9.3		51 (NS)
Yao et al. (2015) NCT00569127	OCT+BEVA	Carcinoids	402	12	16.6	NA
	OCT+IFN			4	15.4	
Libutti et al. (2015) NCT01628913	EVE	pNET	62[a]	9.7	72%	NA
	BEZ235			9.7	70.5% (6 months PFS rate)	
Kulke et al. (2015c) COOPERATE-2 NCT01374451	EVE	pNET	160	6.2	16.8	>34[b]
	EVE+PAS			20.3	16.4	>34[b]
Kulke et al. (2015b) CALGB 80701 NCT01229943	EVE+OCT	pNET	150	12	14.0[c]	35
	EVE+OCT+BEVA			31	16.7	36.7

[a]140 patients planned; [b]not reached; [c]the potential superiority of EB vs E was assessed using a stratified log-rank test with 90% power (1-sided 0.15) to detect a HR of 0.64; [d]if not otherwise indicated.

BEVA, bevacizumab; EVE, everolimus; GEP, gastroenteropancreatic; IFN, interferon-alpha; LAN, lanreotide; NA, not available; NET, neuroendocrine tumor; NS, statistically not significant; OCT, octreotide; PAS, pasireotide; PFS, progression free survival; pNET, pancreatic NET; TTP, time to progression.

patients if an analysis of molecular features would allow an identification of responders in terms of objective response and PFS. In contrast, BEZ235 was poorly tolerated. Treatment was discontinued in 38% of the patients due to side effects compared with 16% with everolimus alone; and the drug will not be further explored. However, novel PI3kinase-AKT-mTOR inhibitors (e.g. PKI-587, a potent novel dual inhibitor of PI3K and mTORC1/C2; inhibitors of mTORC1 and mTORC2, e.g. AZD2014, OSI-027) warrant further investigation based on preclinical studies (Freitag et al. 2016, Massacesi et al. 2016, Vandamme et al. 2016). Another promising combination therapy is metformin and everolimus. Metformin activates adenosine monophosphate-activated protein kinase (AMPK) that is linked with PI3K/PTEN/AKT pathway and MAPK/ERK pathway and might enhance the antiproliferative efficacy of everolimus. Both drugs are currently investigated in pNET along with octreotide (Pusceddu et al. 2014)

Sequential therapies

Although SSA are widely accepted as first-line therapy in SI NET and a subset of pNET, second-line therapy in SI NET might be PRRT or everolimus based on recent results from randomized trials (Strosberg et al. 2015, Pavel et al. 2016, Yao et al. 2016). A population-based retrospective multi-center study from Italy indicated that everolimus use after PRRT and/ or cytotoxic chemotherapy might increase the overall toxicity of everolimus; a smaller retrospective study from the Netherlands, however, reported that the

safety profile of everolimus is unaffected by prior PRRT (Panzuto et al. 2014, Kamp et al. 2013). Sequence of therapies in individual patients may be driven by tumor changes over time. Some tumors may dedifferentiate to high-grade neoplasms requiring cytotoxic chemotherapy (Paul et al. 2016); in contrast, in tumors responding to therapies (either chemotherapy or targeted drugs) the grade/ proliferative activity may decrease (Yao et al. 2008b) or growth factors may be downregulated (von Marschall et al. 2003), and consecutively, less-toxic therapies might be considered. The SEQTOR trial is a prospective trial investigating the optimal sequence of two therapies in advanced or progressive pancreatic NET and aims to clarify if either starting with everolimus or STZ/5-FU followed by cross-over upon progression is superior with respect to DCR and OS (NCT02246127, www.clinicaltrials.gov). Biomarkers will be explored in a subgroup of patients in this trial to identify predictors of response.

Maintenance therapies

The current concept of cancer therapy follows either a continuous therapy as with molecular targeted drugs (e.g. everolimus or sunitinib) or a defined treatment period of 3–12 months with cytotoxic chemotherapy. To prolong the time to progression after termination of cytotoxic therapies or after withdrawal of targeted drugs to limit toxicity, less toxic drugs could be considered. The ongoing placebo-controlled REMINET trial (NCT02288377, www.clinicaltrials.gov) investigates

the role of lanreotide after up to 6 months of prior chemotherapy or targeted agents in pNET.

Novel and future therapeutic approaches

The molecular basis of NET is still poorly understood. With exome and whole genome sequencing and integrated genomic analysis, there is a remarkable increase of knowledge about genetic/epigenetic alterations in intestinal, pancreatic and lung NET. This information may not only pave the way to an integration of molecular data into the classification of NET, that is currently simply based on grading/proliferative activity but also will allow more accurate treatment stratification or even lead to discovery of novel targets.

Mutations in NET of different sites: current knowledge

Pancreatic neuroendocrine tumors (pNET)

Previous understanding of pNET genetics was derived from inherited disorders induced by tumor suppressor dysfunction associated with pNET. The vast majority of pNETs are sporadic, and genes involved in the hereditary disorders have also been attributed to sporadic pNET (Crona & Skogseid 2016). Two studies using next-generation sequencing (NGS) revealed alterations; among those, *MEN1* and *ATRX/DAXX* alterations were the most frequent ones (Table 2; Jiao *et al.* 2011, Yuan *et al.* 2014). The two studies, differing in technology, study population and pNET subtype present varying frequencies for *PTEN, TSC2, TP53* and *KRAS* alterations. A direct comparison of clinicopathological features of pNET from Chinese and US patients revealed higher grade, more advanced stage and larger primary tumor size in Chinese patients, but applicability of WHO and ENETS criteria for both patient groups (Tang *et al.* 2016). Two additional studies (Kimura *et al.* 2016, Vijayvergia *et al.* 2016) confirmed mutations in *KRAS* and *TP53*. PHLDA3, a pleckstrin homology-like protein, which is a potent inhibitor of *AKT* activation (Kawase *et al.* 2009), is inactivated in 72% of pNET patients due to *PHLDA3* gene loss of heterozygosity (Ohki *et al.* 2014).

Small intestinal neuroendocrine tumors (SI NET)

The first genomic analysis of 48 primary SI NET revealed a low mutational burden of 0.1 somatic single nucleotide variants (SSNVs) per 105 nucleotides and a very heterogeneous picture with most alterations found only in individual patients (Banck *et al.* 2013). A parallel study sequencing 55 SI NET again detected only rare recurrent genetic alterations and overlapping genes (e.g. SRC) between both studies were often detected in single patients (Table 2, Francis *et al.* 2013). A detailed investigation revealed recurrent alterations on chromosome 18 affecting *CDKN1* encoding a cyclin-dependent kinase inhibitor regulating cell cycle progression at G1 and confirming previous studies (Kytola *et al.* 2001, Lollgen *et al.* 2001, Kulke *et al.* 2008).

Colorectal neuroendocrine tumors

A comparative investigation focusing on colorectal NEC G3 and colorectal adenocarcinomas unraveled an exceptionally high proportion of *BRAFV600E* hotspot mutations (59%) in NEC G3 including NEC with a signet ring adenocarcinoma component or NEC with conventional adenocarcinoma component (Olevian *et al.* 2016). *KRAS* mutations were only detected in 17% as compared to 43% in the adenocarcinomas. In contrast, another study in high-grade NEC reports BRAF mutations in only 9% (Klempner *et al.* 2016). Thus, colorectal neuroendocrine carcinomas comprise a distinct subgroup within the GI NET, characterized by *BRAF* and *KRAS* mutations, yet in different frequencies compared with colorectal adenocarcinoma.

Impact on prognosis Mutations of *ATRX/DAXX* have been associated with prognosis in pNET; however, conflicting data have been reported on this issue. Marinoni and coworkers identified loss of *ATRX/DAXX* in well-differentiated primary pNET as a negative prognostic marker associated with advanced tumor stage, metastases and reduced survival (Marinoni *et al.* 2014). In line with this, Singhi and coworkers (Singhi *et al.* 2016) identified *ATRX/DAXX* loss as a negative, independent prognostic factor for disease-free survival in more than 300 pNET patients. In contrast, Jiao and coworkers reported an improved survival for patients harboring *ATRX/DAXX* alterations also in combination with *MEN1* mutations (Jiao *et al.* 2011). These contradictory observations most likely stem not only from different sizes of the patient cohorts but also from differently classified pNET. Tang and coworkers used an extended histopathological classification algorithm to distinguish well-differentiated pNET from poorly differentiated NEC of the pancreas (Tang *et al.* 2016). In this analysis, loss of *ATRX/DAXX* was confined to well-differentiated NET, whereas loss of *TP53*, *SMAD4* and *RB* expression characterized poorly

Table 2 Genetic alterations in neuroendocrine tumors.

Study	Genes (%)	Type of alteration	No. of patients analyzed
Pancreatic neuroendocrine tumor			
Jiao et al. (2011)	ATRX (17.6)	NS; MS; indel	10 (WES)
	DAXX (25)	NS; MS; indel	58 (targeted validation)
	MEN1 (44.1)	NS; MS; indel; splice site	
	PIK3CA (1.4)	MS (E545K)	
	PTEN (7.3)	MS; indel	
	TP53 (4.4)	MS; indel	
	TSC2 (8.8)	NS; MS; indel	
	+ 145 genes (>1%)	NS, MS, indel	
Yuan et al. (2014)	ATRX (35.1)	NS; MS; indel	27 (targeted sequencing)
	DAXX (29.7)	NS; MS; indel	
	KRAS (10.8)	MS	
	MEN1 (35.1)	NS; MS	
	PTEN (18.9)	MS; indel	
	TP53 (13.5)	MS; indel	
	TSC2 (43.2)	MS; indel	
	VHL (40.5)	NS; MS; indel	
	SMAD4 (2.7)	MS	
Kimura et al. (2016)	KRAS	MS	1 (individual Sanger seq)
	TP53		
	SMAD4		Immunostaining for TP53 and SMAD4
Ohki et al. (2014)	PHLDA3 (72)	LOH	54 (50 informative) Microsatellite analysis
Vijayvergia et al. (2016)	KRAS (18)	MS	11 NET (targeted sequencing)
	TP53 (18)	MS	
	RB (9)	MS	
	IDH1/2 (9)	MS	
	ATM (9)	Ms	
Small intestinal neuroendocrine tumor			
Banck et al. (2013)	SMAD4 (43)	CNV loss	48 (WES, array CGH)
	AURKA (9)	CNV gain	
	MAP2K4 (18.7)	CNV gain/loss	
	AKT1 (16.6)	CNV gain/loss	
	AKT2 (12.5)	CNV gain/loss	
	PDGFR (16.6)	CNV gain	
	SRC (23)	CNV gain	
	BRAF (6.3)	CNV gain; splice site	
	(many more single alterations)		
Francis et al. (2013)	CDKN1B (10)	Indel	50 (WES; WGS)
	(many more single alterations)		88 for validation
Kim et al. (2016)	BRAF (14.2)	MS	14 (IonAmpliseq Cancer Hotspot Panel NGS)
	SMARCB1 (7.1)	MS	
	RET (7.1)	MS	
	TP53 (14.2)	MS	
	STK11 (7.1)	MS	
	CCNE1 (7.1)	gain	
Colorectal neuroendocrine tumor			
Olevian et al. (2016)	BRAF (59)	MS	32 (Sanger Seq); pure NEC, MANEC and NEC with signet ring cell adenocarcinoma
	KRAS (17)	MS	
Klempner et al. (2016)	BRAF (9)	MS	109 (WES)
	KRAS (32)	MS	
	NRAS (1.8)	MS	
	PIK3CA	MS	

CGH, comparative genomic hybridisation; CNV, copy number variation; indel, small insertion and/or deletion resulting in frame-shift; LOH, loss of heterozygosity; MANEC, mixed adeno-neuroendocrine carcinoma; MS, missense mutation; NS, nonsense mutation; splice site, mutation within a splice site with unknown consequence; WES, whole exome sequencing; WGS, whole genome sequencing.

differentiated NEC, both entities clearly differing in disease-specific survival.

Impact of genomic profiling on therapy
Molecular profiling of NET is currently widely used within clinical trials or as an individualized approach in patients refractory to established therapies. Although the most frequently observed mutations do not present direct drug targets (e.g. *MEN1*, *ATRX/DAXX*), *ATRX/DAXX* loss results in the activation of the alternative lengthening of telomeres (ALT) pathway, a mechanism of telomere maintenance, which may offer new treatment options in the future (Schmitt *et al.* 2016). Even in case potentially druggable mutations have been identified, it remains unclear if they are of functional relevance for tumor growth.

Few studies are reported so far on tumor response in individual patients based on molecular profiling of tumor tissue. In colorectal neuroendocrine tumors, *BRAF* mutations are not routinely tested. Inhibition of BRAF has shown considerable success in melanoma, and combinatorial treatments using BRAF and MEK inhibitors were able to prolong response before resistance in melanomas (Ascierto *et al.* 2016). In two patients with high-grade rectal NEC harboring *BRAFV600E* mutations, treatment with a BRAF-MEK inhibitor combination resulted in a sustained response (Klempner *et al.* 2016) suggesting that BRAF-MEK inhibitors might be an effective therapy for a subset of patients with colorectal NEC. Whole exome sequencing of tumor tissue from 12 GEP-NET patients treated with pazopanib within a clinical trial showed that in 8 of 12 patients, mutations of cancer-related genes, including but not restricted to *TP53, CNBD1, RB1, APC, BRAF, EGFR, KRAS, SMARCB1* and *VHL* were present. Three patients with *TP53* mutations had a durable response to pazopanib, whereas one patient with a small intestinal NET harboring a *BRAF V600E* mutation did not respond to pazopanib (Park *et al.* 2016). The study is limited by the small number of patients including only 3 of 12 patients with progressive disease as best response. Molecular alterations of TP53 have been reported in 4–18% of NET and seem to be associated with more aggressive subtypes of NET. Interestingly, *TP53* mutations upregulate VEGF-A and VEGFR2. In a prospective trial in 188 evaluable patients (56% harbored *TP53* mutations) with advanced or progressive solid cancers *TP53* mutations predicted response to VEGF/VEGFR inhibitors (Wheler *et al.* 2016). These findings may guide future studies with angiogenesis inhibitors in NET.

KRAS mutations are a hallmark alteration of pancreatic adenocarcinoma. Three publications reported low-frequency *KRAS* mutations (10–20%) also in pNET (Yuan *et al.* 2014, Vijayvergia *et al.* 2016, Kimura *et al.* 2016). Although targeting RAS directly is a mission impossible (Cox *et al.* 2014), new options arouse from targeting RAS downstream effectors and cell cycle checkpoint control. In *NRAS* mutant melanomas (Ascierto *et al.* 2016), inhibition of MEK produced a partial response in a subset of patients. Further, inhibition of MEK and CDK4/6 was effective in a preclinical study using patient colorectal xenografts (Lee *et al.* 2016) and thus represents another option for a subset of NET patients harboring *KRAS* or *NRAS* mutations.

Previous small studies with imatinib in NET lacked objective responses or were associated with toxicity (Gross *et al.* 2006, Kindmark *et al.* 2010). In a study with 27 patients, one patient with partial response to imatinib was reported (Yao *et al.* 2007). However, in a patient with an activating mutation within the *cKIT* gene indeed a sustained response to imatinib was achieved (Perkins *et al.* 2014), thus imatinib may be considered an option in highly selected patients with *cKIT* mutation.

Mutations in the mTOR pathway occur in up to 14% of pNET. In an *in vivo* NET model and in patients treated with everolimus and octreotide, an increased AKT activation was associated with rapamycin sensitivity (Meric-Bernstam *et al.* 2012). In an exploratory biomarker analysis in more than 500 patients with human EGFR 2-positive advanced breast cancer, it could be demonstrated that patients with cancers showing *PIK3CA* mutations, PTEN loss or hyperactive PI3K pathway derive PFS benefit from everolimus (André *et al.* 2016). Biomarker analysis in subsets of patients from previous everolimus trials and from ongoing trials with mTOR pathway inhibitors will provide more insight into their predictive value in the future. Interestingly, in patients with recurrent glioma strong mutation-inducing capacity of temozolomide was reported. Exome sequencing of recurrent tumors revealed alterations in both the mTOR (*MTOR, AKT, PTEN* a.o.) and RB (*RB, CDK4,6, CDKN2A*) pathway (Johnson *et al.* 2014). Similar events might be ongoing in NET, with potential impact on sequencing of therapies and support re-biopsy as a valuable tool for directing treatment.

Biomarker-driven therapies

The lack of real-time biopsies is a major limitation to exploit and validate potential biomarkers for therapy selection. In an ongoing phase 2 trial in GEP-NET, patients will be assigned to a targeted therapy based on a mutation profile determined in tumor tissue

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either retrieved at surgery or from a biopsy. Patients will be treated with sunitinib (for mutations in *MEN1/PDGFR/cKIT/FLT3*) or everolimus (for mutations in *NF1/PTEN/PI3K/AKT/mTOR/VHL/TP53*) (Neychev *et al.* 2015). This mutation-targeted therapy will elucidate if a more specific therapy stratification based on biomarkers will be associated with a more durable response.

Epigenetics in NET

Due to the lack of a 'genetic driver-signature' in most NETs, epigenetic alterations became a research focus and provided potentially prognostic and predictive biomarkers. Epigenetic modifications include DNA methylation, histone modifications and miRNAs. Recently, NET epigenome profiling has led to the identification of molecularly distinct tumor subsets in pNET and SI NET (reviewed in Cives *et al.* 2016, Stålberg *et al.* 2016).

The most comprehensive analysis integrating transcriptome (mRNA and miRNA), metabolome and selected mutations in pNET was provided by Sadanandam and coworkers (Sadanandam *et al.* 2015). A miRNA and mRNA expression analysis was performed on 51 human pNET samples and in a mouse model of pNET (RipTag2). Three subgroups of patients were identified with distinct metastatic behavior and metabolic features. The islet/insulinoma tumor (IT) subtype comprises tumors with low grade and low metastatic potential. The metastasis-like primary (MLP) subtype was enriched for genes associated with fibroblasts/stroma, stem cells and hypoxia and found in many metastatic samples. The intermediate subtype (the only one not found in the RipTag2 samples) was composed of non-functional pNET, a subgroup of which 16% was associated with distant metastases. This type harbored by far the majority of *MEN1* and *ATRX/DAXX* mutations.

Similarly, in SI NETs that are characterized by significant epigenetic dysregulation in 70–80% of tumors, three subgroups of intestinal NET with different outcomes and PFS rates were identified based on an integrated genomic, epigenomic and transcriptomic analysis (Karpathakis *et al.* 2016, Stålberg *et al.* 2016). Features of the group with the best prognosis included one of the most prevalent genomic alterations in intestinal NET, the LOH of chromosome 18 and *CDKN1B* mutations; a second group with intermediate prognosis was associated with the presence of frequent promoter methylation of tumor suppressor genes (so called CpG island methylator phenotype) and the absence of copy number variations; the third group with the worst prognosis showed multiple copy number variations. Although this molecular division of pNET and SI NET reflects well the heterogeneity of NETs observed in clinical practice, like with most other gene expression profiles, independent validation of the signatures is necessary to determine its impact on patient prognosis and potential use for therapy stratification.

Although epigenetic clustering has prognostic value in pNET and SI NET, it needs to be further explored how far epigenomic profiling may provide reliable predictive biomarkers. The enzyme O6-methylguanine DNA methyltransferase (MGMT) is a DNA repair enzyme removing alkyl-guanine adducts after chemotherapy with alkylating agents, such as streptozotocin or temozolomide. The reported frequencies of *MGMT* promoter methylation (PCR or pyrosequencing) range between 0 and 40%, and for MGMT expression, between 24 and 51% depending on the method and study (Liu *et al.* 2016). Promoter hypermethylation seems to be associated with response to temozolomide in some studies; however, prospective validation is lacking (Schmitt *et al.* 2014, Walter *et al.* 2015). Two ongoing prospective trials, one in GEP-NET (temozolomide+lanreotide, NCT02231762) and one in pNET (temozolomide vs temozolomide+capecitabine, NCT01824875) included MGMT expression and promoter methylation as a biomarker.

Epigenetic modifications can be reversed and thus represent a potential target for novel drug therapy such as inhibition of enzymes involved in DNA methylation and histone modification. Although epigenetic drugs (histone deacetylase (HDAC) inhibitors e.g. valproic acid, panobinostat and DNA methyltransferase inhibitors azacytidine, decitabine) generated promising results in NET cell lines (Alexander *et al.* 2010, Arvidsson *et al.* 2016), the few studies with HDAC inhibitors in NET patients performed so far lacked major responses. In a phase II study in 15 patients with GEP-NETs, no objective remissions were observed, but stable disease was achieved as best response with panobinostat in all patients (Jin *et al.* 2016). The DNA methylation and deacetylation inhibitor, RRx-001, is the only epigenetic drug under clinical investigation to sensitize high-grade neuroendocrine tumor patients who previously responded and now have failed to a platinum-based chemotherapy (NCT02489903, www.clinicaltrials.gov).

Transcriptome analyses

A more pathway-oriented approach in the understanding of these complex and heterogeneous tumors became

apparent through transcriptomic approaches, and first data indicate that molecular profiling may guide treatment decisions (Cutler *et al.* 2016). Studies from Missiaglia and coworkers (Missiaglia *et al.* 2010) reported reduced expression of TSC2 and PTEN in a significant number of pNET samples (60%) and an association of low TSC2/PTEN levels with lower survival and shorter TTP. These results, however, were challenged by other studies that failed to demonstrate a prognostic impact of TSC2 or PTEN (Qian *et al.* 2013, Haugvik *et al.* 2016). In contrast, high expression of mTOR or its activated downstream targets was associated with worse prognosis (Qian *et al.* 2013). Craven and coworkers (Craven *et al.* 2016) identified pancreas-specific angiogenesis transcriptional signatures. *FGFR* and *VEGFR1-3* gene were upregulated in pNET and PDAC (pancreatic adenocarcinomas). On the contrary, *FGF9* (fibroblast growth factor 9), *ANGPTL3* (angiopoietin-like 3), *SCG2* (secretogranin 2)

among others were specifically high in pNET. Angiopoietin-like 3 and secretogranin 2 are involved in endothelial cell migration, vessel formation and survival; FGF9, a ligand of the FGFR, correlated with EGFR inhibitor resistance in colorectal cancer (Mizukami *et al.* 2016). This indicates a clear rationale for the antiangiogenic therapy in pNET, which is based on gene expression rather than gene mutation. Targeting multiple angiogenic pathways that take into account mechanisms of escape and resistance seems to be a more promising approach (Fischer *et al.* 2007, Yao & Phan 2011, Sennino *et al.* 2012). In this respect, several TKIs targeting VEGFRs and FGFRs are currently under clinical investigation (Table 3). The complex pattern of pNET and PDAC-specific angiogenic markers suggests different mechanisms of response and resistance, which need to be underpinned by more detailed biomarker analyses (Jayson *et al.* 2016), which may have an impact on the choice of antiangiogenic drugs in the future.

Table 3 Signaling pathways and novel drugs in clinical trials in neuroendocrine tumors.

Pathway/targets	Drugs	Mechanism(s)	Clinical trial design	Study
PI3K	SAR245409 (XL-765)	PI3K/mTOR inhibitor	Phase I in solid tumors	NCT00485719
	CC-223	mTOR1+mTOR2-inhibitors	Phase I/II in solid tumors[a], MM, NHL	NCT01177397
	BYL791+everolimus	PI3K inhibitor	Phase Ib in pNET	NCT02077933
	GDC-0941	PI3K inhibitor	Phase I in solid cancers	NCT00876109
	LY2584702+everolimus	p70S6K inhibitor	Phase I in patients with solid tumors[b]	NCT01115803
RAF/MEK/ERK	Regorafenib	VEGFR1-3, c-KIT, TIE-2, PDGFR-β, FGFR-1 RET, RAF-1, BRAF and p38 MAPK inhibitor	Phase II in pNET/carcinoids	NCT02259725
Retinoblastoma	LEE011 (ribociclib)	CDK inhibitors	Phase 2 in lung and GEP NET (foregut)	NCT02420691
	PD0332991 (palbociclib)		Phase 2 in pNET with overexpression of Cdk4 and/or phospho-Rb1 and/or cyclin D1	NCT02806648
VEGFR/FGFR/PDGFR	Ramucirumab+SSA	VEGFR-2 antagonist	Phase II in carcinoids	NCT02795858
	Nintedanib	VEGFR1-3, FGFR 1-3 PDGFR α-/ β-inhibitor	Phase II in carcinoids/NEN	NCT02399215
	Sulfatinib	VEGFR1-3, FGFR1- inhibitor	Phase III in pNET, Phase III in carcinoids	NCT02678780
	Famitinib	c-Kit, VEGFR2+3, PDGFR, Flt1 and Flt3-inhibitor	Phase II in GEP NET	NCT01994213
	Lenvatinib	VEGFR1-3, FGFR1-4, RET, c-kit, PDGFRα	Phase II in pNET/GI NET	NCT02678780
	Cabozantinib	VEGFR-2, c-Met inhibitor	Phase II in pNET, carcinoids	NCT01466036
	X-82+Everolimus	VEGFR/ PDGFR inhibitor	Phase II in pNET	NCT01784861
	Ziv Aflibercept	VEGF-A, VEGF-B, and PLGF trapping	Phase II in carcinoids	NCT01782443
	Ziv-Aflibercept+Sapanisertib	VEGF/ PLGF trapping+mTOR1- and-2 inhibition	Phase I in solid tumors[c]	NCT02159989
Ubiquitin-proteasome	SNX 5422+Everolimus	Heat-shock protein 90 (Hsp90) inhibitor	Phase I in NET	NCT02063958
	Carfilzomib	Proteasome inhibitor	Phase II in advanced NET	NCT02318784

[a]includes non-pancreatic NET; [b]includes advanced NET; [c]includes pancreatic NET; NHL, non Hodgkin lymphoma; MM, malignant melanoma.

Expression profiling also revealed novel targets such as the cyclin-dependent protein kinase CDK4 which was overexpressed in around 60% of pNET tissues. CDK4 and 6 are involved in phosphorylation of the retinoblastoma (Rb) tumor suppressor gene leading to its inactivation. Growth of a human pancreatic cell line (QGP1) was inhibited in a xenograft mouse model by a CDK4/6 inhibitor (PD0332991), which reactivates the Rb pathway (Tang *et al.* 2012). These findings paved the way for further exploration of CDK inhibitors in NET (Table 3).

Heat-shock protein 90 (Hsp90) is a molecular chaperone that has been shown to play an important role in the stabilization and activation of numerous key oncogenic client proteins. Several of the proteins that are known to be overexpressed in GEP-NET are regulated by Hsp90, including EGFR, ERBB2, IGF-1R and AKT. Preclinical data indicate that Hsp90 is a promising target for growth inhibition in NET (Gloesenkamp *et al.* 2012, Fendrich *et al.* 2014); SNX-5422, an Hsp90 inhibitor is currently investigated in a phase I trial with everolimus in advanced NET (NCT02063958, www.clinicaltrials.gov).

Improving our understanding of the complexities of interaction of pathways

Current targeted therapies in NET include a broad spectrum of inhibitors directed against tumor-driver pathway such as PIK3CA/mTOR, MAPK and RTKs (EGFR, FGFR, PDGFR and VEGFR) (Table 3). Although in selected patients mutations may become predictive markers of response and epigenetic profiling may guide therapy, a mechanistic explanation for response or resistance is lacking for the majority of inhibitors. Furthermore, concepts such as dual inhibition of pathways to prevent signaling network-intrinsic feedback responses have been tested only to a limited extent. Chiu and coworkers (Chiu *et al.* 2010) tested a combinatorial therapy of the mTOR inhibitor rapamycin together with the EGFR inhibitor erlotinib in the RipTag2 mouse model. The strong effect that rapamycin exerted in this model was enhanced by combining rapamycin and erlotinib and lead to an increased survival of tumor-bearing mice. This analysis comprised a bona fide example of how combinatorial treatments might enhance the suppression of a pathway. However, erlotinib is an EGFR inhibitor with maximal effectiveness in *EGFR* mutant (T790M) non-small cell lung cancer (Barton *et al.* 2010), whereas in most neuroendocrine tumors, neither high level EGFR expression nor mutations have been observed

(Kidd *et al.* 2013). In a preliminary analysis of a phase II study of everolimus and erlotinib in NET patients ($n=17$), no remissions were observed in the subgroup of 9 patients with carcinoids, whereas 7 patients had stable disease; pNET data were not reported, and further use was limited by toxicity (Bergsland *et al.* 2012).

Thus, signaling networks and components such as RTKs relevant in subsets of NET have to be identified to enable a rational combinatorial therapy and understand obvious resistance mechanisms. Kolch and coworkers summarized major principles of signaling dynamics, network regulation and its impact onto cancer cell proliferation and drug resistance (Kolch *et al.* 2015). A mechanistic model relevant for NET is the feedback from mTORC to the adaptor protein IRS1, intimately connected with RTKs such as EGFR and IGF1R. This feedback results in AKT activation but might also result in increased MEK-ERK activation (via IRS-stimulated RAS activation) and provide an escape mechanism during everolimus therapy. Network wiring from RTK to PIK3CA/mTOR and MEK/ERK signaling is reasonably well understood; however, the influence of genetic alterations found in neuroendocrine tumors, including *TSC2, AKT* and *PTEN* alterations onto network behavior during therapeutic interference is unknown and is currently explored in NET (http://www.sys-med.de/de/demonstratoren/maptor-net). Recently published experimental work provided novel ideas on how network behavior in the context of specific genetic alterations can have an influence on therapy (François *et al.* 2015, Schwartz *et al.* 2015, Soler *et al.* 2016, Xu *et al.* 2016). Selective inhibition of PIK3CAβ in PTEN-deficient cancers results only in a transient suppression of AKT/mTOR activity due to feedback-dependent activation of RTKs and subsequent activation of PIK3CAα (Schwartz *et al.* 2015). On the contrary, PIK3CAα inhibition in tumors harboring RTK or PI3K mutations resulted in PIK3CAβ activation. Only dual inhibition of both isoforms resulted in sustained pathway suppression and inhibition of tumor cell survival. Also, Soler and coworkers describe both a growth-suppressive and angiogenesis-suppressive effect of the selective p110 PIK3CA inhibitor (GDC-0326) in the RipTag2 mouse model for pNET upon combination with the pan-PI3K inhibitor (GDC-0941) (Soler *et al.* 2016). Although only selected papers are mentioned here, it becomes clear that for successful targeted therapy in NET, a much deeper understanding of pathway wiring, feedback control and role of the deregulated genes is urgently required. Furthermore, clinical trials with

both broad and selective inhibitors will provide data on distinct efficacies (Massacesi *et al.* 2016).

Immunotherapy in neuroendocrine tumors

Immunotherapy is a rapidly evolving field in different types of cancer. Checkpoint blockade therapy targeting cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the programmed cell death protein 1 pathways (PD-1/PD-L1) is being associated with dramatic tumor responses in different types of cancer including lung cancer, kidney cancer and melanoma. In advanced Merkel cell carcinoma, a rare neuroendocrine virus-linked skin cancer, pembrolizumab, an anti–PD-1 monoclonal antibody was associated with an objective response rate of 56% (Nghiem *et al.* 2016).

In NET, the anti-tumor immune response is antagonized by several tumor-related factors. Tumor-associated antigens and cytokines from NET cells, dendritic cells and tumor-associated macrophages, induce and recruit regulatory T cells, which inhibit the anti-tumor immune response (Ameri & Ferone 2012). Few patients with NET have been treated so far with checkpoint inhibitors. Best response was stable disease in a patient with carcinoid and a patient with pancreatic NET (Patnaik *et al.* 2015), and it remains unclear which NET patient subgroups might potentially benefit from this therapy. In light of the activity of pembrolizumab in small-cell lung cancer and Merkel cell carcinoma (Horn *et al.* 2016, Nghiem *et al.* 2016), the role of immunotherapy might be particularly relevant in high-grade NEC.

The Keynote-158 Study (NCT02628067, www. clinicaltrials.gov) is currently investigating pembrolizumab in multiple types of advanced solid tumors including carcinoids that have progressed on standard of care therapy, and other trials are under construction. There are limited and inconsistent data in NET on molecular markers that have been associated with response to checkpoint inhibitors such as PD-1 or PDL-1 staining in tumor tissue and stroma. In one study in neuroendocrine carcinoma (61 of pulmonary, 33 of extrapulmonary origin), PD-1/ PDL-1 staining was only found in the microenvironment, macrophages and not on tumor cells (Schultheiss *et al.* 2015). Another study in foregut and hindgut tumors reported an expression of PDL-1 in 22% of tumor tissues and was associated with worse survival, but higher response rate to chemotherapy (Kim *et al.* 2016). It is currently a matter of debate which biomarkers may best predict response to checkpoint inhibitors. Mutational burden, composition and activity of a preexisting immune infiltrate and

mechanisms of tumor escape from immune surveillance are considered important components associated with the probability of response (Dijkstra *et al.* 2016).

The use of dendritic cells represents a more individualized approach of immunotherapy. Tumor vaccination has been tried in endocrine malignancies many years ago, however, with negative results (Papewalis *et al.* 2008). In the recent years, this approach has been modified and successfully explored in melanoma. It is likely that with this approach a clinical benefit will be achieved rather by combining personalized dendritic cell-based vaccination with additional strategies (Datta *et al.* 2015)

Another approach of cancer immunotherapy is the use of oncolytic viruses engineered to selectively kill tumor cells. An engineered oncolytic adenovirus (AdVince) has been developed for the treatment of liver metastases from NET. The adenovirus includes the gene promoter from human chromogranin A for selective replication in neuroendocrine cells, and a cell-penetrating peptide in the capsid for increased infectivity of tumor cells, and will be injected in the liver (Leja *et al.* 2011, Yu *et al.* 2016). A clinical trial is currently recruiting patients with NET (RADNET; NCT02749331).

Conclusions

From small trials in heterogenous types of NET with origin in various organs the NET field moved toward well-constructed placebo-controlled trials in well-defined patient populations, mainly separated in trials for pancreatic NET and non-pancreatic (intestinal and/or lung) NET. Evidence-based trials led to approval of molecularly targeted drugs including somatostatin analogues, sunitinib and everolimus. Placebo-controlled trials provided more insight into the highly variable spontaneous tumor growth behavior of NET. The exploration of targeted drugs in a broad range of NET revealed that some NETs share commonalities with respect to drug sensitivity irrespective of their primary site. Potentially druggable mutations are low in NET and still their functional relevance needs to be validated. With increasing knowledge of the molecular background of NET and definition of distinct molecular subgroups even in patients with NET of the same site of origin, the outcome of patients might be improved by individualized stratification of patients to specific treatments in the near future. The exploration and validation of biomarkers in ongoing trials will complement all ongoing endeavors in the field of

genomics and epigenomics, as well as the focus on hallmarks of cancer so far widely unexplored in NET.

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